1. INTRODUCTION AND AIMS

Behavioural toxicology is an emerging field, becoming increasingly important in risk assessment of exposure to neurotoxic substances. This is due to the high sensitivity of behavioural towards neurotoxic action, and to the integration of several underlying processes and neural functions - motor, sensory, attentional, motivational - in behavioural phenomena.

The neurotoxicity of many xenobiotics, including organic and inorganic forms of heavy metals, has been identified on the basis of their adverse effects observed on humans' and laboratory animals' brain functions.

In the neurotoxicological works of the Department it was found that heavy metals, including inorganic forms of lead (Pb), Mercury (Hg) and manganese (Mn), induced marked alterations in the spontaneous and stimulus-evoked activity of the rat central nervous system (CNS) (Nagymajtényi et al., 1997; Schulz et al., 1997; Papp et al., 2000; 2005; Pecze et al., 2002; Vezér and Papp, 2002; Vezér et al., 2000a; 2000b; Vezér et al., 2001; 2005; Pecze et al, 2004a,b). These alterations may represent, or be concomitant to, changes of plastic representation at the cortical and subcortical level (Stoltenburg-Didinger, 1994). As the cortical representation of external stimuli is a crucial element of behavioural mechanisms, it was reasonable to include behavioural methods in the investigation of environmental xenobiotics, to have a more comprehensive access to the toxic effects and the underlying mechanisms.

Abnormalities in the higher order functions of the CNS are important aspects of the damage caused in humans by exposure to heavy metals. In case of lead, learning difficulties constitute the main symptom in humans (ATSDR, 1999a; Needleman, 1990; Winneke, 1994). Lower IQ of lead exposed children, compared to non-exposed controls, was described by Schwartz, 1994. In case of adults suffering occupational Pb exposure, the outcome was less clear (Goodman et al., 2002). In rats, chronic exposure to inorganic lead resulted in impaired learning and decreased hippocampal long-term potentiation (LTP) (Altmann et al., 1993). In an earlier experiment of the Department, Pb acetate caused significant decrease of open field (OF) activity (Nagymajtényi et al., 1997). In later studies (Vezér et al., 2000a; 200b) both Pb-acetate and tetraethyl-lead (the lead compound added to petrol in Hungary up to 1990) caused

significant loss in the memory performance of adult rats in maze tasks and marked motor hypoactivity.

Mercury causes human behavioural abnormalities in both inorganic and organic forms (ATSDR, 1999b). In animal experiments, alterations of T-maze behaviour (Zenick, 1974) and locomotion (Rossi et al., 1997) were seen on administration of methyl mercury, the form mostly responsible for population exposure. Our own experiments indicated similar behavioural effects of inorganic (Vezér et al., 2001; 2002) and organic (Vezér et al., 2005) forms of Hg.

Our above-mentioned works made it clear that combination of the methods available at the Department – including recording and analysis of cortical electropysiological signals (Papp, et al., 2000; Papp et al., 2005) – allow state-of-the-art investigation of heavy metal effects on higher order CNS functions in animal experiments. At the same time, environmental data in the literature indicated that other metals deserve to be included into neurobehavioral experimentation, and one of these was manganese.

Manganese is, in small amounts, an essential micronutrient, a cofactor in metallo-enzymes. In prolonged exposure to higher doses it is, however, a potential environmental neurotoxicant. In the general population, excess dietary intake (beyond the estimated safe and adequate daily amount) is typical Mn as man-made environmental contaminant originates from Mn-containing waste (e.g. batteries), methylcyclopentadienyl manganese tricarbonyl (MMT) used as anti-knock petrol additive, and organo-Mn agricultural fungicides (ATSDR, 2000).

Brain is among the primary target organs in chronic Mn exposure (Roels et al., 1987), and the turnover of Mn there is much slower than in other parts of the body (Feldman, 1992). Mn was shown to cause childhood hyperactivity disorder (Barlow, 1983), manganese psychosis, motor deficit (Mergler et al., 1999); as well as altered childhood psychomotor development (Takser et al., 2003) and impairments of several memory process (list learning, visual recognition, digit span).

Similar deficits were also induced by Mn in animals (T-maze learning: Öner and Sentürk, 1992; motor hypoactivity: Ingersoll et al., 1995; altered acoustic responsivity: Dorman et al., 2000). In these works, however, behavioural effects were not supported by

histochemical or electrophysiological findings, and no follow-up in the after-exposure period was included.

Hence, our experiment involved a complex behavioural test battery (8-arm radial maze, OF activity, acoustic startle response – ASR, and pre-pulse inhibition – PPI); and the behavioural investigations were supplemented by Mn level determination in blood, cortex and hippocampus (HC), and by histological examinations. All investigations were performed both during and after the period of Mn administration. It was also attempted to prove the involvement of the dopamine (DA) system by applying a dopaminergic agonist in the elimination period.

The questions to be answered in these experiments were as follows:

- What alterations are induced by oral Mn administration in the learning and memory processes, spontaneous exploratory activity, and acoustic startle response?
- How long does it take for the alterations in higher order CNS functions to appear, and are these constant or increasing during the period of exposure to Mn?
- Is there any detectable increase in the blood Mn level, and deposition of Mn in peripheral tissues and CNS structures responsible for the behavioural effects?
- Are the Mn-induced behavioural alterations reversible upon cessation of the metal administration?
- After cessation of Mn administration, can behavioural effects, indicating altered plasticity, be elicited by means of decreasing cortical inhibition using the indirect dopaminergic agonist d-amphetamine (d-A)?

2. BEHAVIOUR AND NEUROTOXICITY

2.1. Structural and functional background of the investigated behavioural phenomena

2.1.1. Learning and memory. Cognitive behaviour

Numerous studies demonstrated the role of the HC in memory (Olton and Papas, 1979; Shapiro, 2001; Lee et al., 2005). HC is in a strategic position to process information derived from the polymodal association areas of the neocortex and to redistribute it to cortical and subcortical regions involved in the modulation of complex behavioural processes. It is the most critical brain area for ability to recollect everyday events and factual knowledge.

Intrahippocampal connections form a trisynaptic loop; composed of the granule cells of dentate gyrus (DG) (afferent), CA3 and CA1 pyramidal cells (efferent) and their interconnections (Stoltenburg-Didinger, 1994) (Fig. 1).

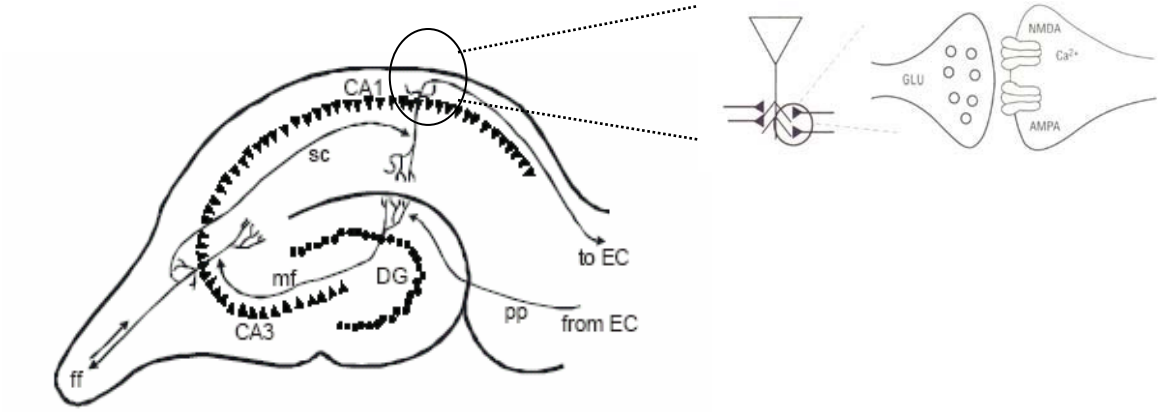


Figure 1 The anatomy of the hippocampus (adapted from Amaral and Witter, 1989; Stoltenburg-Didinger, 1994)
Left: the trisynaptic loop. Abbreviations: EC: entorhinal cortex; DG: dentate gyrus; pp: perforant pathway; mf: mossy fibers; Sc: Schaffer collaterals; ff: fimbria fornix.
Right: a single CA1 pyramidal neuron and the hippocampal synapse. NMDA and other (e.g. AMPA, kainate) receptors are colocalized. Simultaneous glutamate binding to NMDA receptors and postsynaptic depolarisation leads to calcium influx. This dual gating of the NMDA receptor provides a mechanistic explanation for many of the induction properties of long-term potentiation (LTP).

The perforant pathway (pp in Fig. 1) provides the HC its main glutamatergic input. Collaterals of the same axons form also connections with CA3 pyramidal cells. A major afferent system to the CA3 pyramidal cells are the mossy fibres, the axons of DG cells. Mossy fibres also have synapses with the mossy cells of the DG, providing feedback excitation to the granule cells. The axons from the CA3 pyramidal neurons form glutamatergic synapses on the

CA1 pyramidal cells (Schaffer collaterals). These axons pass through the fimbria/fornix. Other pathways that pass via the fimbria/fornix are noradrenergic connections from the locus coeruleus, serotoninergic pathways from the raphe nuclei, histaminergic connections from supramamillary nucleus and dopaminergic one from ventral tegmental area and the substantia nigra (Dutar et al., 1995). Both inhibitory cholinergic projections (ascending connection between the septum and HC) and the γ-amino butyric acid (GABA) to GABA input (interneurons of the HC) provide a synchronous orchestration of the entire hippocampal formation (Chrobak, 2000).

Electrophysiological recordings from freely moving rats showed that some CA1 and CA3 hippocampal pyramidal cells fire only in a restricted part of the environment, the so-called "firing field" of the cell. This observation, together with the fact that the hippocampal damage causes dramatically deficit on rats' spatial learning (Morris et al., 1982), led to hypothesis that the HC implements an abstract representation of the environment, called a cognitive map (Banquet et al., 2005). Beside the CA1 and CA3 cells, also the input-area of the HC (DG and lateral septum) and the output area (subiculum) show location-selective firing (Quirk et al., 1992; Zhou et al., 1999; Sharp and Green, 1994), furthermore the pre- and post-subiculum contains so-called head-direction cells (Muller et al., 1996). These cells provide more information for the hippocampal formation that computes the location and position of the animal.

Glutamate (Glu), the primary excitatory neurotransmitter in the CNS, binds to various receptors in the HC including (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA). When NMDA receptors are activated, calcium enters the cell (MacDermott et al., 1986) and triggers a cascade of events. An important property of the hippocampal circuity is that synaptic efficacy can be enhanced through repeated stimulation. Repeated high frequency stimulation at the NMDA receptor results in long-term potentiation. The LTP has evolved to a basic model of memory (Shapiro, 2001).

In the wild, spatial learning and memory help animals find locations that provide food and safety, and therefore are crucial foe survival. Learning and memory are not measured directly in experimental settings, but they are inferred indirectly from changes in behaviour. Learning is regarded as enduring change in behaviour while memory is the preservation of

that learning over time. The very important problem of separating memory effects of a drug from effects on other factors of behaviour, like sensory processes, attention and motivation of the subject, was addressed by Heise (1984).

Rats, displaying extraordinary spatial abilities, are commonly used in studies examining cognitive capacity of animals in spatial tasks, realized in a variety of mazes. Banquet et al. (2005) postulated that the rats can solve maze tasks by using at least three different learning strategies, each representing a different learning ability. One of them, referred to as place learning, is spatial learning occurring in relation to all kinds of available distant cues. This type of learning requires formation of a map of the environment. The strategies used by rats to find food in, e.g., a partially baited radial maze, are thought to reflect foraging strategies used by these animals in their natural habitat. For efficient task solving, rats must employ a win-stay strategy between foraging sessions, based on a long-term reference memory. Within a foraging session, they have to employ a win-shift strategy based on a short-term or working memory (Beatty and Shavalia, 1982). Radial arm maze, with a subset of rewarded arms, is thus a useful tool to dissociate the spatial reference and spatial working memory components.

2.1.2. Open field exploratory behaviour

Exploration is a result of the interaction between elements in the space (external elements, representing the physical parameters of the environment like light, food, predators etc.) and in the organism (internal elements, representing the neural-physiological structure and actual state of the organism). Exploratory behaviour is complex and dynamic, due to the instability of the environment and the rapid interactions between the organism and the world surrounding it. OF behaviour, the natural manifestation of spatial learning (Biegler and Morris, 1996; Banquet et al., 2005), has been traditionally considered as complex and stochastic (Tchernichovski and Golani, 1995). OF exploratory behaviour of rats consists of regular excursions into the environment from a preferred place termed a „home base”. With time, there is a gradual increase in the length of the excursions (Tchernichovski and Benjamini, 1998). The rats perform increasingly longer paths from one location, and exploration is more extensive at the proximal part of the route, and less at the distal part

(Tchernichovski et al., 1998). In a novel environment, rats usually run to the nearest wall or relatively massive object before exploration. If such a feature is absent the rats do not explore but simply keep running. According to Mataric (1994a, 1995) the existence of some massive object is crucial for exploratory behaviour to occur.

In the higher order exploratory system, locomotion is directed to distal stimuli that comprise the features of an environment (Lammers et al., 1988). Sinnamon (1993) suggested that the various locomotor nuclei/regions could be classified according to the behavioural context in which they serve to initiate locomotor activity. He differentiated three types of locomotion in the freely-moving animals: an „exploratory", a „primary appetitive" and a „primary defensive" system (Fig. 2).

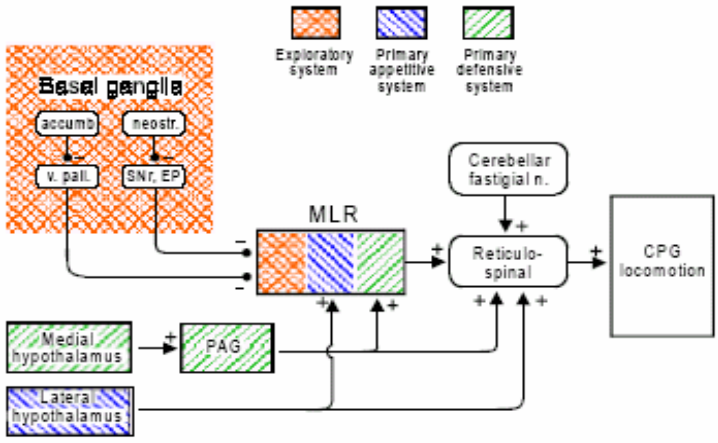


Figure 2 Brainstem structures important for initiation of locomotion in mammals. The basal ganglia nuclei included are the nucleus accumbens (accumb), the ventral pallidum (v. pall.), the neostriatum (neostr.), the substantia nigra (SNr), and the entopeduncular nucleus (EP). The components of the exploratory system are distinguished by the hatched patterns. Inhibitory connections are illustrated by filled circles, and excitatory connections are represented by arrowheads (Modified from Grillner et al., 1997).

2.1.3. Acoustic startle response and pre-pulse inhibition

The ASR reaction is a contraction of the skeletal and facial muscles in response to an abrupt, intense (>80 dB) auditory stimulus (Koch, 1998). ASR is understood as a protective response to unexpected, aversive events; and is based, in rats, on a relatively simple, reflex-like mechanism. The magnitude of the ASR can be modulated by various external and internal (emotional states) conditions. The reflex is very fast, the onset latency of actual muscular

responses is 5 to 10 ms in rats (Pellet, 1990). By measuring this reflex, effects of an injury to the brain or other parts of the neural system can be detected. The magnitude of the ASR elicited by a given signal is reduced when a weak, non-startling sensory event precedes the startle-eliciting signal by an appropriate lead interval. This phenomenon is termed PPI. Suppression of the ASR with a weak, non-startling pre-stimulus it is used as a model for sensorimotor gating processes (Koch, 1998).

The short latency of the ASR only allows for few synaptic relays interposed between the cochlea and the motor neurons. In the last 15 years different various models have been proposed using on the one hand from three to five relays, on the other hand focusing on functional aspects or on neuroanatomical structures. Nowadays, Koch and Schnitzer's model scheme (1997) is generally accepted (Fig. 3).

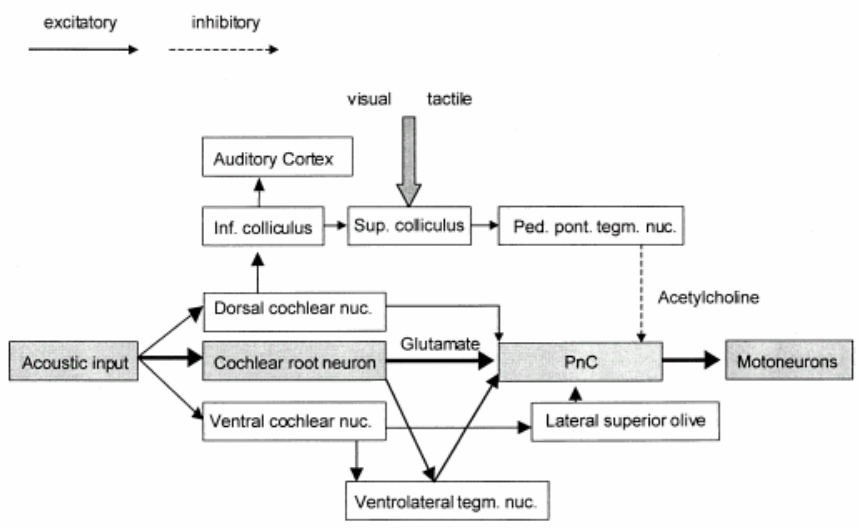


Figure 3 Hypothetical pathways mediating the acoustic startle reflex (ASR) and pre-pulse inhibition (PPI) (modified from Koch, 1998). Shaded areas are the main components of the basic startle reflex. The PPI pathway includes the inferior and superior colliculi and the pedunculopontine tegmental nucleus (PTTg). The caudal pontine reticular formation (PnC) is regarded as a central interface between the ASR and PPI pathways. In the model showed, the primary startle circuit consists of five neural substrates in rats: auditory nerve, ventral cochlear nucleus, nuclei of the lateral lemniscus, nucleus reticularis pontis caudalis, spinal interneurons, plus the neuromuscular junction.

2.2. Neurotoxic mechanisms in manganese exposure

An over-exposure to Mn is known to lead to toxic manifestations including neurotoxicity. Population-level Mn exposure is typically due to excess dietary intake via drinking water (Kondakis et al., 1989), or food (Iwami et al., 1994). Another source of exposure can be the increasing Mn level of the environment (ATSDR, 2000).

A number of reports described the association of human low-level chronic Mn exposure and various neurological manifestations. Hyperactivity and delayed psychomotor development was found in children (Crinella et al., 1998; Takser et al., 2003), and Parkinson-like symptoms or "idiopathic Parkinson's disease", in adults (Gorell et al., 1997; Youdim et al., 1993). The described neurological deficits possibly result from the so-called manganese encephalopathy (Mergler et al., 1999) - with Mn deposition in the basal ganglia, particularly in the globus pallidus and striatum (Mergler, 1996) or substantia nigra and HC (Lucchini et al., 1999). In animal experiments, excess Mn was found to deposit in mitochondria-rich tissues including the liver, pancreas, pituitary gland, and in the muscles (Barceloux, 1999). Within the brain, highest Mn level was detected after subchronic administration in the cerebral and cerebellar cortex (Dorman et al., 2000); and in case of chronic ingestion, in the pons and medulla (Takeda et al., 1998), but lowest in the hypothalamus (Chan et al., 1992).

There is a certain parallelism between neuropathology in human Parkinson's disease, the morphological changes obtained in animals by 6-hydroxy dopamine (selective dopaminergic toxin; Ponzoni et al., 2000) and the functional alterations caused by Mn^{2+}. Subchronic oral administration of $MnCl_2$ (50 mg/kg b.w.) to neonatal rats resulted in increased pulse-elicited acoustic startle reaction amplitude, and in increased striatal DA and 3,4-dihidroxyphenylacetic acid level (Dorman et al., 2000). Chronic Mn exposure also led to hepatic encephalopathy, due to hyperammonaemia (Chandra and Shukla, 1978), corresponding to increased substrate supply for synthesis of the amine neurotransmitters DA, noradrenalin (NA), serotonin (5-HT) and histamine (Bergeron et al., 1989). Release of the other neurotransmitters, e.g. Glu and GABA, was reduced by Mn^{2+} in moderate doses (Takeda et al., 2002), but excess Mn^{2+} also reduces the conversion of Glu to glutamine, a process localized primarily in the astrocytes and performed by the Mn-dependent glutamine synthetase (Aschner et al., 1999). Elevated extracellular Glu could result in NMDA/Glu

receptor mediated neurotoxicity (Butterworth, 1998). This excitotoxicity could play a key role in Mn-induced neuronal cell death (Hazell, 2002), or degenerative astroglial changes in the globus pallidus (Baeck et al., 2003).

The hippocampal formation is under central cholinergic inhibitory modulation (Metherate et al., 1992). The muscarinic cholinergic system can be affected in Mn exposure (Pappas et al., 1997). Damage to the cholinergic projection by other means (damage to the bilateral fimbria-fornix: Whishaw and Jarrard, 1995; or selective lesioning of the basal forebrain cholinergic system: Leanza et al., 1995) was shown to cause impaired cognitive behaviour.

In the present study, the points of aims stipulated in Chapter 1 were addressed by behavioural tests aimed at the cognitive (spatial learning and memory task in an 8-arm radial maze), and affective (putatively dopaminergic: acoustic startle response, and cholinergic: startle plasticity) effects of Mn. These behavioural results were supplemented by histological investigations of the HC, and by Mn level determinations in two regions of the brain and in peripheral tissue samples.

3. METHODS

3.1. Subjects and housing

Young adult (8 weeks old, body weight 220-250 g) male Wistar rats (strain code: Crl:[WI]BR) were obtained at the University's Breeding Center. The animals were kept – during the 2 weeks acclimation period and during the whole course of treatments and tests – in polypropylene home cages (up to 4 rats/cage) and maintained in a controlled environment (12/12 hour light/dark cycle with light on at 6:00 a.m.; temperature 22 to 24°C). The rats had unlimited access to drinking water, their access to food was controlled as required by the experimental protocol.

The time course of the experiments is shown in Table 1.

Table 1 Tests performed during the pre-treatment, treatment and post-treatment periods in the experiments. The post-treatment period (shaded) was done only with $MnCl_2$. The numbers in the cells indicate the number of animals used in the tests.

Periods	Weeks	Maze learning	OF	ASR and PPI	Histology	Tissue sampling
Pre-treatment	0th	-	(3 x 12)	-	-	Blood (3 x 3)
Treatment	1st	Adaptation (3 x 16)	-	-	-	-
	2nd	Acquisition (3 x 16)	-	-	-	-
	3rd	WM 2 h (3 x 16)	-	-	-	-
	4th	RM (3 x 16)	-	-	-	-
	5th	WM 4 h (3 x 16)	(3 x 12)	-	-	Blood, HC, cortex, etc. (3 x 3)
	6th	Rest (3 x 13)	-	-	-	-
	7th	Rest (3 x 13)	-	-	-	-
	8th	Return (3 x 13)	-	-	-	-
	9th	WM R 2 h (3 x 13)	-	-	-	-
	10th	WM R 4 h (3 x 13)	(3 x 12)	(3 x 12)	GFAP-IR	-
Post-treatment (elimination)	11th	Rest (3 x 6)	-	-	-	-
	12th	Rest (3 x 6)	-	-	-	-
	13th	Return (3 x 6)	-	-	-	-
	14th	WM R 2 h (3 x 6)	-	-	-	-
	15th	WM R 4 h (3 x 6)	-	-	-	-
	16th	-	-	-	-	-
	17th	-	(3 x 6)	(3 x 6)	-	-
	18th	-	0.5 d-A (3 x 6)	-	-	-
	19th	-	-	-	-	-
	20th	-	1.5 d-A (3 x 6)	-	-	-
	21st	-	-	-	-	-
	22nd	-	-	-	-	Blood, HC, cortex, etc. (3 x 6)

Abbreviations: GFAP-IR, glial fibrillary acidic protein-immunoreactivity; WM, working memory; RM, reference memory; Rest, rest period; WM R, repeated working memory; d-A, d-amphetamine; HC, hippocampus; OF, open field; ASR, acoustic startle reaction; PPI, pre-pulse inhibition.

The experiments were started with 48 rats, distributed randomly to three groups (16 animal/group) by their body weight in the 0^{th} week. After the second week of acclimation, one group received the high dose (59 mg/ kg b.w., 1/25 LD_{50}) and another, the low dose (15 mg/kg b.w., 1/100 LD_{50}) of the metal studied, for 10 weeks (treatment period) in a 5 days per week scheme, by gavage. The substance ($MnCl_2 \cdot 4H_2O$, min. 99% purity, REANAL Hungary) had a per os LD_{50} of 1484 mg/kg b.w. (Physical and Theoretical Chemistry Laboratory, 2001). $MnCl_2$ was dissolved in distilled water to an administration volume of 1.0 ml/kg. The control group received distilled water. In the post-treatment period, no more metal was given in order to see the effects of a possible elimination.

3.2 Behavioural tests

3.2.1. Spatial working and reference memory testing

The animals' spatial learning and memory ability was tested in an 8-arm radial maze (Columbus Instruments, Ohio, USA). The maze was kept in a constant position in the middle of a square room with two doors and no windows. All furniture (desk, chairs, shelf etc.) was maintained in the same position during the experiment. The illumination of the room was 25 lux, and 40 dB white background noise was present. Throughout the spatial memory tests, the animals were food deprived maintaining them at 80-85% of their free-feeding body weights, adjusted for growth (Beatty and Shavalia, 1982). Rat food was available ad libitum from Friday to Sunday evening, and on the other days, for 1 hour per day after the tests.

Short term working and reference memory tests

The short term memory tasks were performed in the 1^{st} to 5^{th} treatment weeks (see Table 1). In the first week of treatment (adaptation), all animals had a 10-minute training twice a day, adapting them to find food pellets in the maze arm ends. Completion the radial maze involved the rat running down each of the 8 arms branching from the octagonal centre platform, in order to obtain the food reward at the end of each arm of the maze. Perfect performance of this task required entering each arm only once. During the 2^{nd} week, with one training per day, the rats were first individually trained to learn the general cues of the task,

that is, entering each one of the 8 arms only once in a given session, with no more than one error per session in 6 consecutive days (acquisition of the task). Acquisition errors consisted of revisiting an arm previously visited in the same session. This way, the rats were forced to learn a win-shift food search strategy (Beatty and Shavalia, 1982). The percent rate of correct responses was counted as:

(correct responses - acquisition errors) x 100,

and was taken as performance indicator. All animals achieved a run performance of over 85%. Successful solution of the maze task requires the rats to recall the arms already visited, mainly when the performance is interrupted (Olton et al., 1977). In conducting one of the tests in the radial maze, delays of variable intervals can be introduced between the 4th and the 5th arm choice (Bartus et al., 1985). The introduction of these intervals permits the precise testing of the skill of the rats in remembering the arms previously visited in one session (working memory, information required in the actual session only), and by varying the length of these intervals an indication of the short term memory performance can be obtained.

Short term spatial working memory tests

They were done in the 3rd and 5th treatment weeks, that is, after successful acquisition. The rats (all 16 per group) were one by one put for 10 min maximum in the centre of the maze, but were allowed to enter only 4 of the 8 open and baited arms (at their own random selection); this was the "event-to-be-remembered". After visiting the four arms, the animal was returned to its cage and kept there for 2 or 4 hours (WM 2 h and WM 4 h in Table 2). The rat was then put again in the maze centre and allowed to complete arm choices 5-8 to obtain rewards in the 4 baited arms not visited before. In the working memory tests, errors (WM error) meant re-entry into any of the arms visited in the first run. Working memory performance was thus counted as (correct responses - WM errors) x 100.

Reference memory

It was tested in the 4th treatment week with 16 rats per group. Here, food reward was put only in the 4 arms preferred by the individual rats. Entering an unbaited arm constituted a reference memory error, from which performance was calculated as above.

Rest periods

In the 6^{th}-7^{th} treatment weeks, the animals were kept in the housing room, were treated with $MnCl_2$, but did not have any testing and were not exposed to new information. In the 1^{st}-2^{nd} post-treatment weeks, there was another rest period without $MnCl_2$ administration.

Long term working memory tests

The long term memory was tested in the 8-10^{th} treatment weeks and, repeatedly, in the 3^{rd}-5^{th} post-treatment weeks. In the 8^{th} treatment week, memory return was observed (with 13 rats per group), and in the 9^{th} and 10^{th} treatment week, 2- and 4-hours working memory (WM R 2h, WM R 4h in Table 1). This sequence was repeated (with 6 rats per group) in the 3^{rd}-5^{th} post-treatment weeks.

3.2.2. Spontaneous locomotor activity in the open field

The rats' spontaneous motor behaviour was assessed, using a fixed subset of 12 rats from each group, in the 0^{th}, 5^{th} and 10^{th} week of treatment phase (Table 1). From each group, 6 (previously tested) rats had the OF test in the 17^{th} week, and in the 18^{th} and 19^{th} week after a challenge by d-A. By inducing release of DA from presynaptic endings, d-A acts as an indirect $DA_{1/2}$ agonist and can elicit stereotyped behaviour (gnawing, sniffing, repetitive head and body movements) and hyperactivity (Costall et al., 1977).

The test was performed always once in the given weeks, following the maze-learning session. The animals were first allowed to accommodate in the testing room for 30-40 minutes. An OF box of 40x40x40 cm size (ACTIFRAME, Gerb Electronic, Berlin, Germany), equipped with two arrays (at 3 and 15 cm above floor level) of infrared movement detectors with 1.1 cm distance between the beams, was used. Illumination at the floor of the OF was 12-25 lux, with ca. 40 dB white background noise. The animals were placed into the centre of the box and their spontaneous horizontal (running), vertical (rearing) and local (predominantly grooming) activity was scored during a 10 min session. Movement scores were computed on the basis of beam interruptions with a PC software having preset conditions to discriminate between forms of movement.

3.2 3. Psychomotor performance and startle plasticity testing

Acoustic startle response

In this test, assessing a putative dopaminergic sensorimotor control (Koch, 1999), the contraction of the whole body musculature as a response to a sudden auditory stimulus (>80 dB) is measured. ASR of the rats was tested in the 5^{th} and 10^{th} treatment week during Hg and Mn exposure (12 animals/group), and in the 7^{th} post-treatment week (6 animals/group) of the Mn experiment, by means of a commercially available reflex monitor (Responder X System, Columbus Instruments, Ohio, USA). To measure ASR, the rats were placed individually on a metal plate, mounted on a piezoelectric accelerometer and surrounded by a Plexiglas test cage (16x28x18 cm), located inside a sound and light-attenuated chamber (100x80x60 cm). Movements of the rats resulted in changes of the voltage output of the accelerometer, which was amplified, digitized, and fed into the computer of the Responder X System for further analysis. The presentation of the acoustic stimuli was controlled by the same computer. A loudspeaker mounted 10 cm from the cage delivered a continuous white background noise (~65 dB_{RMS}) throughout the session, and also the acoustic startle stimuli. After a 10-min accommodation, the rats received a series of 10 consecutive tones (5 kHz, 110 dB, 200 ms, 15 s intervals) as test stimuli. A whole body twitch resulting in more than 50 g force to the cage floor was accepted as “noise positive response”, of which latency, time to peak and peak force amplitude were measured; the latter calculated from the difference between the peak-to-peak voltage output of the accelerometer within time windows of 80 ms after and 80 ms before the startle stimulus onset.

Startle plasticity test

PPI is the normal suppression of the startle reflex when the intense stimulus is preceded by a weak non-startling pre-stimulus. It has been viewed as an operational measure of sensorimotor gating mechanisms of the brain, activated to reduce information procedure interference in sensory and motor systems (Braff et al., 1992). In our experiment, startle plasticity of the animals was tested together with the ASR, in a second series following 15 min rest. This time, the startling test pulses were preceded by inhibiting (non-startling) pre-

pulses (1 kHz, 73 dB, 500 ms, 200 ms delay between pre-pulse and startling pulse), similarly to the method published by Graham (1975).

3.3. Tissue manganese level determination

3.3.1. Blood collection

Blood samples were collected several times in the pre-treatment, treatment, and post-treatment (Mn only) periods, as shown in Table 1. The tail vein was punctured and blood was taken to heparinized vacutainers of 7.0 ml capacity (BD367735, Becton-Dickinson). All blood samples were stored at 4 ^{o}C until analyzed.

3.3.2. Cortex, hippocampus, liver, kidney and femur sampling

In the 5th and 10th treatment weeks, and in the 12th post-treatment week, after finishing the behavioural tests, 3, 5 and 6 animals per group, respectively, were sacrificed with an overdose of Nembutal. The thorax was opened and the animals were transcardially perfused with 500 ml saline to remove blood from the organs. The brain was removed whole, without the meninges, and was, under a stereomicroscope, halved and dissected to isolate the HC. The whole hippocampus, 0.3 g of the cortex, liver and kidney, and the distal part of the femur (without the periosteum) was taken as sample, and kept at -18 °C until analyzed.

3.3.3. Manganese level determination

The samples were digested in 2 ml concentrated nitric acid, with 0.5 ml hydrogen peroxide added for HC, cortex, liver, kidney and femur. Total manganese concentration (μg/g) in the samples was determined by flameless graphite furnace atomic absorption spectrometry at the accredited laboratory of the National Centre for Public Health, Budapest.

3.4. Immunhistochemistry

After finishing behavioural tests in the 10th week (Table 1), 2 rats per group were brought to terminal Nembutal anaesthesia and were perfused transcardially first with 50 ml physiological saline (pre-rinse) then with 400 ml 4% paraformaldehyde fixative solved in 0.1M phosphate buffer (PB, pH 7.4). The brains were removed from the skull, postfixed for 4h and cryoprotected by overnight storage in 30% sucrose in PB. Coronal sections were cut on a cryostat microtome at a 50 μm thickness and collected in phosphate buffered saline.

Immunhistochemical reactions were performed on free floating sections. After abolishing endogenous tissue peroxidase activity by treatment with 0.6% H_2O_2 in Tris buffered saline (TBS; 0.1M, pH 7.4), the sections were incubated in 5% normal goat serum (NGS) in TBS containing 0.1% Triton X-100 for 1 hour. Subsequently, tissues samples were incubated in monoclonal anti-glial fibrillary acidic protein (GFAP, an indicator of gliosis due to neuronal damage) (Sigma, 1:10000 in TBS containing 0.1% Triton X-100, 5% NGS) for 16 hours at room temperature. The sections were thoroughly rinsed and exposed to biotinylated goat anti-mouse IgG (Dianova, 1:300 in TBS containing 5% NGS) for 1 hour. Finally, sections were processed with preformed complexes of streptavidin and biotinylated peroxidase and stained with nickel-enhanced 3,3'-diaminobenzidine as a chromogen.

The density of GFAP-IR was determined for the hippocampal CA1 stratum radiatum and stratum oriens, and for the hilus of the DG.

The area of GFAP-IR structures was determined by video imaging using an Image Pro Plus 4.0 image analysis software (Media Cybernetics, Silver Spring, Md, USA). Stained sections were examined under bright field with an Olympus microscope and a 10x objective. Images were recorded with a SONY 950-P CCD camera (Sony Corp., Japan) and digitized. The program expressed the area innervated by the IR as number of pixels having densities above the threshold. Measurements were taken in a blinded fashion from at least 16 sections for each animal group and averaged.

3.5. Body and organ weight measurements

Body weight of the rats was measured in the first 11 weeks of the experiment on each workday, and weekly averages were calculated. In the 2^{nd} to 13^{th} post-treatment weeks, weighing was done on Mondays only, at the same time of the day, and two-weekly averages were calculated to reduce standard deviation at low number of animals and high inter-individual variations.

The whole study was done in adherence to the requirements by the Ethical Committee for the Protection of Animals of the University.

3.6. Statistical analysis

The distribution of the data (except the ASR/PPI scores) was checked for normality by the Kolmogorov-Smirnov test. Depending on the distribution, the statistical analyses were carried out by MANOVA or Kruskal-Wallis one-way ANOVA for the spatial learning and memory tests, one-way ANOVA for the ASR/PPI measured data, and two-way ANOVA for body weight and total manganese contents. In case of MANOVA, post hoc analysis of group differences was performed by Scheffe's or by Duncan's test in case of body weight. Group comparisons following the Kruskal-Wallis test were performed by the Mann-Whitney U test. The number of animals giving "noise positive" response in the ASR and PPI procedure was evaluated with the Chi^2 test. In all cases, the confidence level was set to $p<0.05$.

For statistical analysis, the software package Statistica for Windows 4.0 was used.

4. RESULTS

4.1. Manganese tissue concentrations

4.1.1. Blood manganese

Total Mn level in the tail vein blood in the 0th (pre-treatment) week, 5th and 10th treatment weeks, and 2nd, 6th and 12th post-treatment weeks are given in Table 2. In the 5th and 10th $MnCl_2$ treatment weeks, blood Mn levels in the high dose (59 mg/kg b.w.) group showed significant increase compared to untreated control in the same week (5th week: $F_{2;6} = 12.16$; high dose vs. control and vs. low dose: $p<0.01$; 10th week: $F_{2;6} = 9.51$; high dose vs. control and vs. low dose: $p<0.01$) and also compared to the 0th week values. Likewise, in the 2nd and 6th post-treatment weeks, only the group treated previously with the high dose had significant dose dependent elevation of blood Mn level ($F_{2;6} = 12.16$; high dose vs. control: $p<0.01$).

At the end of the experimental period (12th post-treatment week) blood Mn level in the treated rats was nearly identical to that of the control group or that measured in the pre-treatment (0th) week.

Table 2 Manganese content (μg/g) of the blood in the pre-treatment (0th) week, in the 5th and 10th treatment week, and in the 2nd, 6th and 12th post-treatment week. Mean±SD.

Mn (μg/g) in blood	The whole experimental period (0th–22nd experimental weeks)					
	Pre-T	Tp		p-Tp		
	0th week	5th week	10th week	2nd week	6th week	12th week
	n=3	n=3	n=5	n=3	n=3	n=6
Control	0.01	0.03±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01
15 mg/kg	±0.01	0.05±0.01	0.02±0.01	0.01±0.01	0.02±0.01	0.02±0.01
59 mg/kg		0.32±0.14** ##	0.04±0.02** ##	0.02±0.01*** ##	0.03±0.01** #	0.02±0.01

Abbreviations: Pre-T, pre-treatment week, Tp, treatment period; p-Tp, post-treatment period; n, number of the animals measured per group.
**: $p<0.01$, ***: $p<0.001$ treated vs. control group
#: $p<0.05$, ##: $p<0.01$ high dose group vs. low dose group

4.1.2. Tissue manganese

Mn levels of all tissue samples collected and analysed are given in Table 3. Significant increase of tissue Mn levels was seen only in the group receiving the high dose. In the 5th treatment week, Mn levels in the kidney ($F_{2;6} = 5.34$; high dose vs. control and vs. low dose: $p<0.05$) and femurs ($F_{2;6} = 46.72$; high dose vs. control, and vs. low dose: $p<0.001$) was significantly elevated. By the 10th treatment week, Mn levels in the cortex ($F_{2;12} = 5.22$; high dose vs. control and vs. low dose: $p<0.05$), hippocampus ($F_{2;12} = 9.41$; high dose vs. control and vs. low dose: $p<0.01$), liver ($F_{2;12} = 4.66$; high dose vs. control and vs. low dose: $p<0.05$), kidney ($F_{2;12} = 5.25$; high dose vs. control and vs. low dose: $p<0.05$), and femur ($F_{2;12} = 5.73$; high dose vs. control, and vs. low dose: $p<0.05$) were all significantly higher in the high dose $MnCl_2$-treated than in the control group.

In the 12th post-treatment week, there was no more significant difference between the Mn levels of cortex and HC in the control and treated rats. In the kidney and femur, however, Mn level in the high dose group was still significantly and dose-dependently elevated (kidney: $F_{2;15} = 24.41$; high dose vs. control and vs. low dose: $p<0.001$; femur: $F_{2;15} = 16.33$; high dose vs. control and vs. low dose: $p<0.001$). In the liver, Mn levels were significantly elevated also in the low dose group ($F_{2;15} = 23.62$; high dose vs. control: $p<0.001$; high dose vs. low dose: $p<0.01$; low dose vs. control: $p<0.01$).

The drop of cortical and hippocampal Mn level during elimination was stronger in the high dose than in the low dose group, which phenomenon was paralleled by certain functional parameters.

Table 3 Manganese content of the cortex, hippocampus, liver, kidney and femur samples taken 5th and 10th treatment week; and 12th post-treatment week - elimination. Mean±SD.

Periods	Week of periods	Doses (mg/kg)	Mn (ug/g) in				
			Cortex	Hippocampus	Liver	Kidney	Femur
Tp	5th (n=3)	Control	0.22±0.03	0.17±0.01	2.25±0.31	0.79±0.07	0.35±0.01
		15 mg/kg	0.21±0.03	0.26±0.09	2.48±0.25	0.88±0.28	0.37±0.03
		59 mg/kg	0.28±0.03	0.23±0.06	3.22±0.71	1.38±0.29*#	0.62±0.06*** ###
	10th (n=5)	Control	0.31±0.02	0.46±0.04	2.18±0.24	0.75±0.14	0.35±0.04
		15 mg/kg	0.32±0.06	0.47±0.05	2.57±0.21	0.70±0.15	0.38±0.05
		59 mg/kg	0.44±0.11*#	0.68±0.14** ##	3.98±1.68*#	2.28±1.47*#	0.69±0.29**#
p-Tp	12th (n=6)	control	0.31±0.03	0.59±0.10	1.65±0.14	0.54±0.07	0.26±0.03
		15 mg/kg	0.31±0.06	0.60±0.17	2.13±0.37**	0.54±0.04	0.29±0.05
		59 mg/kg	0.29±0.03	0.57±0.13	2.71±0.24***##	0.78±0.08*** ###	0.52±0.14*** ###

Abbreviations: Tp, treatment period; p-Tp, post-treatment period, n, number of the animals measured/group.
*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ treated vs. control group
#: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$ high dose group vs. low dose group

4.2. Spatial learning and memory

During acquisition (2^{nd} week of treatment), in the short term 4 hours working memory (5^{th} treatment week) and reference memory (4^{th} treatment week), and in all of the long term retention tests (9^{th}-10^{th} treatment and 4^{th}-5^{th} post-treatment weeks), both $MnCl_2$-treated groups showed, compared to control animals, a significant, dose dependent decrease in the average memory performance, as seen in Fig 4.

During all phases of the maze learning test (i.e., on the 2^{nd} to 5^{th}, 8^{th} to 10^{th}, and 13^{th} to 15^{th} weeks) both $MnCl_2$ treated groups showed, compared to control animals, a decrease in the average memory performance (Fig. 4).

Acquisition (2^{nd} week) was dose-dependently impaired in the $MnCl_2$ treated rats ($F_{2;15} = 7.51$; high dose vs. control: $p<0.01$; low: $p<0.05$). Reference memory (4^{th} week) showed in both treated groups a significant performance deficit ($F_{2;12} = 24.68$; high dose vs. control, $p<0.001$; low vs. control, $p<0.01$; high vs. low dose, $p<0.05$). During the 4 hours short-term working memory tests (5^{th} week) the memory performance of the treated rats was significantly and dose-dependently lower than in the controls ($F_{2;9} = 16.85$; high dose vs. control, $p<0.001$; low vs. control, $p<0.05$; high vs. low dose, $p<0.05$). After 2 weeks rest period, the control and low dose group both showed a memory return to the level seen on the 2^{nd} week but the level reached by the high dose animals was ca. 20 % less. In the long-term retention test (9^{th} week) both $MnCl_2$ treated groups showed a further significant memory deficit vs. control ($F_{2;12} = 26.14$; $p<0.001$) and the high vs. low difference was also significant ($p<0.001$). On comparison of the long-term (9^{th} and 10^{th} week) and short-term (3^{rd} and 5^{th} week) memory retention it was found that the group receiving low dose Mn had a better performance in the 9^{th} than in the 3^{rd} week. All other differences were insignificant.

At the end of the 10^{th} week, Mn administration was finished. After further two weeks (13^{th} week), memory return was greatly improved in the high dose group compared to the 8^{th} week. In the long-term retention tests, however, no noteworthy change in any of the treated groups was seen.

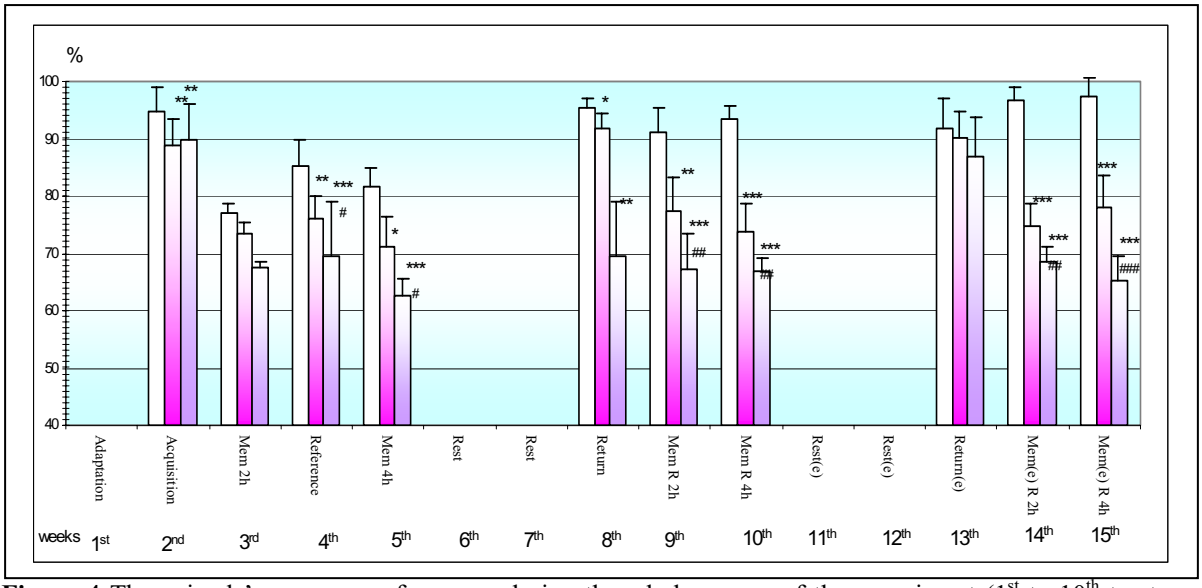


Figure 4 The animals' memory performance during the whole course of the experiment (1st to 10th treatment week and 1st to 5th post-treatment week). Performance (see Methods for calculation) is given as weekly average of group averages (mean±SD; n=16/group in the 1st-5th treatment weeks and 13/group in the 6th-10th treatment weeks, and 6/group from then on).
Abbreviations: Mem, working memory; Mem R, repeated working memory; (e) indicates the phase of post-treatment period.
*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ treated groups vs. control group
#: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$ high dose group vs. low dose group

4.3. Open field activity

In the OF activity, a general decreasing trend was observed which was due to the aging of the animals. Irrespective of that, $MnCl_2$ treated animals had decreased spontaneous activities.

Horizontal activity of the treated animals decreased in the 5th and 10th week in a dose- and time-dependent manner vs. untreated (or vs. 0th week) controls (Fig. 5A; $F_{4;72} = 11.04$; 5th week: both doses vs. control, $p<0.01$; 10th week: both doses vs. control, $p<0.05$). The interaction of dose and treatment time was not significant ($F_{4;72} = 2.17$; n.s).

Decrease of the local activity (Fig. 5B) became significant by the 10th week ($F_{4;72} = 3.18$; both doses vs. control, $p<0.01$). Interaction of doses and treatment times: $F_{4;72} = 1.98$; n.s.

The number of rearings (Fig. 5C) was reduced in a dose-and time-dependent manner vs. controls by the 5th week ($F_{4;72} = 7.19$; both doses vs. control, $p<0.001$; interaction: $F_{4;72} = 2.17$; n.s.). In the 10th week, a near-control level of vertical activity was seen.

After 7 weeks without treatment (17^{th} week) the difference between the control and treated groups was minimal in all three forms of activity. One week later (18^{th} week) 0.5 mg/kg d-A was given ip. and OF activity was tested 15 min later. Locomotor activity increased to some extent but the effect was moderate and not significantly different in the treated and control animals (compared to results of 17^{th} experimental week). Allowing two weeks for d-AM to disappear, this test was repeated on the 20^{th} experimental week with a higher dose (1.5 mg/kg). Here, local activity (Fig. 5B) decreased in both treated groups ($F_{2;12} = 5.42$; low dose vs. control: $p<0.01$, high dose: n.s). Horizontal activity (Fig. 5A) showed an increasing trend in both groups vs. control but the effect was significant only in the low dose group ($F_{2;12} = 4.15$; $p<0.05$). The high dose group also showed clearly (albeit not significantly) increased vertical activity after 1.5 mg/kg d-AM (Fig. 5C).

4.4. Psychomotor performance and sensorimotor gating

4.4.1. Acoustic startle response

In the 5^{th} treatment week, the effect of Mn on the number (Fig. 6A) and measured parameters (onset latency, peak time, peak amplitude) of the noise positive responses was below significance.

At the end of treatment period, Mn dose-dependently decreased the number of acoustic responses given on 10 consecutive stimuli ($Chi^2 = 16.63$, $p<0.01$). In the 7^{th} post-treatment week, the difference in the number of noise positive responses was, compared to 10^{th} treatment week's results, not significant in any of the groups while the reaction of the treated rats was again significantly different from the controls ($Chi^2 = 9.75$, $p<0.05$). The alterations in the measurable parameters of the noise positive ASR responses in the 10^{th} treatment and 7^{th} post-treatment week were moderate (Fig. 6B, C). Onset latency increased significantly in the 10^{th} treatment week in both treated groups vs. control ($F_{2;24} = 4.06$; $p<0.05$ for both groups vs. control), but in the 7^{th} post-treatment week, only the high dose group showed significantly lengthened latency ($F_{2;14} = 8.20$; high dose vs. control: $p<0.01$; high dose vs. low dose: $p<0.05$).

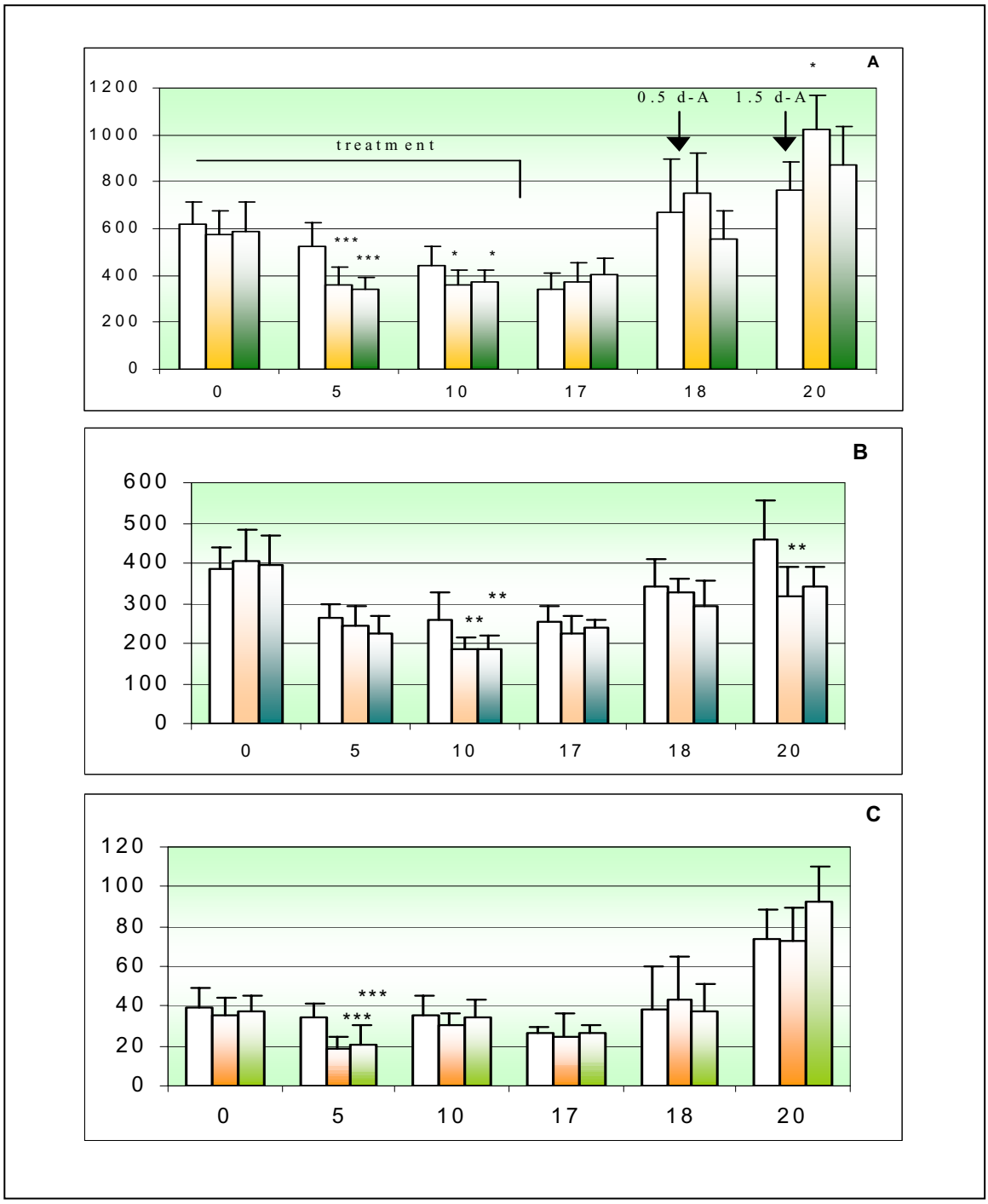


Figure 5 Effects of $MnCl_2$ on the spontaneous and d-amphetamine (d-A)-induced OF activity of the animals during the treatment and post-treatment period. ***A***: Horizontal (length run, cm), ***B***: local (grooming; counts) and ***C***: vertical (rearing, counts) activity was measured during a 10 min OF session.
Mean±S.D., n= animals sampled in each group.
*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ treated groups vs. control group

The peak amplitude of the ASR responses decreased in both $MnCl_2$-treated groups but the change was not significant and was stronger in the low than in the high dose group (not shown).

4.4.2. Pre-pulse inhibition on the ASR

In the 10th treatment week, the effect of the PPI was significantly reduced in both treated groups (especially in the low dose group) vs. control (Fig. 7A). It, in fact, was changed to facilitation, i.e. there were more responses than without pre-pulse ($Chi^2 = 22.98$, $p < 0.01$). In the 7th post-treatment week, the effect of the pre-pulse on the ASR response was nearly identical in the $MnCl_2$-treated and control groups ($Chi^2 = 0.55$, $p>0.05$). PPI acted paradoxically also on the measured ASR parameters. The latency decreased ($F_{2;23} = 10.01$; low dose vs. control: $p<0.001$; high dose vs. control: $p<0.01$; Fig. 7B) and the peak time shows an increasing trend (Fig. 7C). After 7 weeks in the post-treatment period, this effect has not become more expressed.

4.5. Body weight and relative organ weights

4.5.1. Body weight

On the same animals in each group (control, 12; low and high dose, 10 per group: altogether 32) body weight was measured daily (5 day/week) during the 10 weeks of treatment (Fig. 8A); and on 6 animals per group (altogether 18) weekly during the 12 weeks of post-treatment period (Fig. 8B).

In the 6th to 10th treatment weeks, the weekly average body weight in the high dose group was significantly reduced compared to the controls ($F_{2;12} = 7.19$; high dose vs. control: $p<0.01$), and after the 8th treatment week, also vs. low dose group ($F_{2;12} = 18.16$; high dose vs. control: $p<0.001$, high vs. low dose: $p<0.01$). In the low dose group, the body weight deficit was significant vs. control in the 8th, 9th and 10th treatment weeks ($F_{2;12} = 12.36$; vs. control: $p<0.05$).

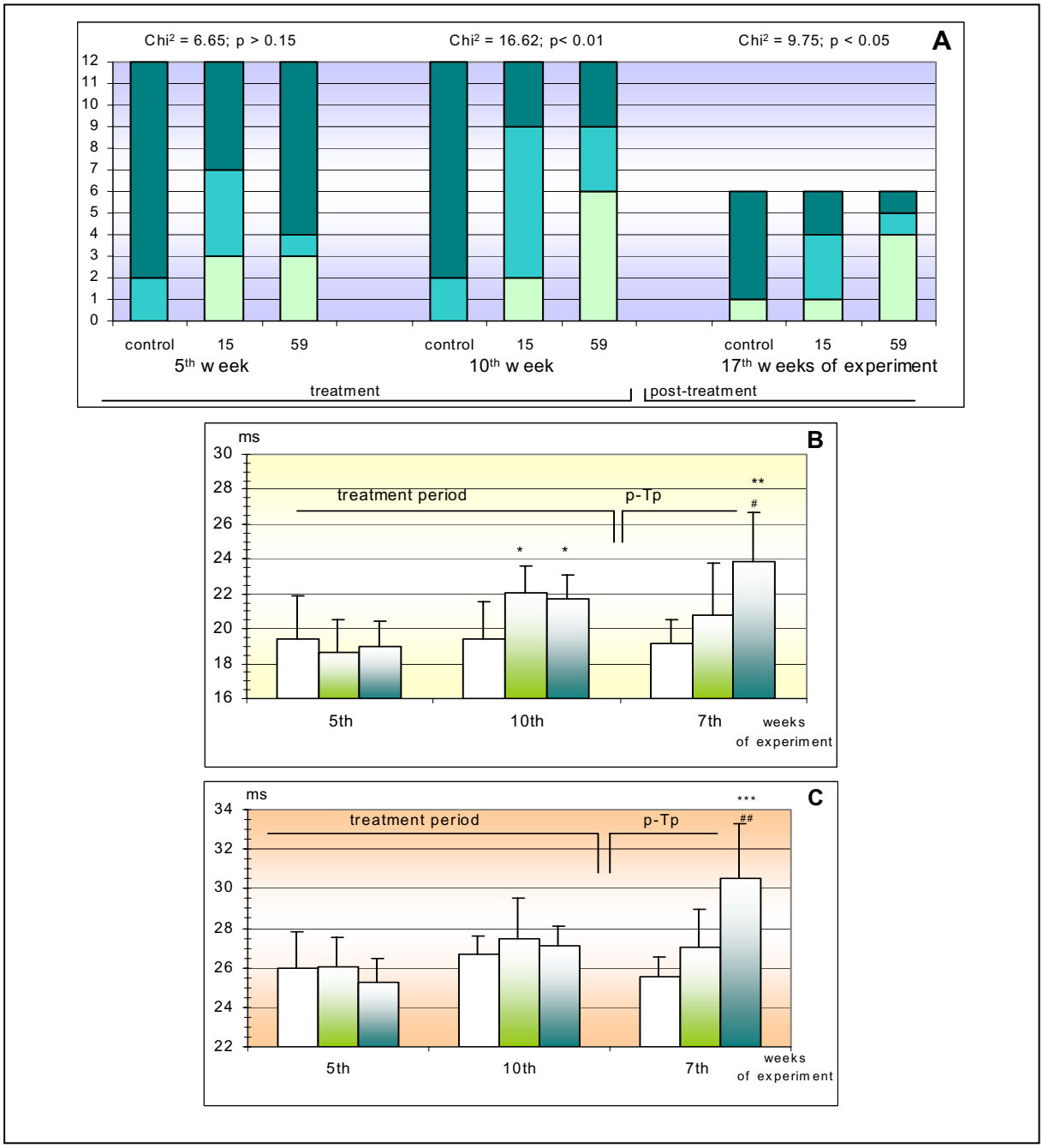
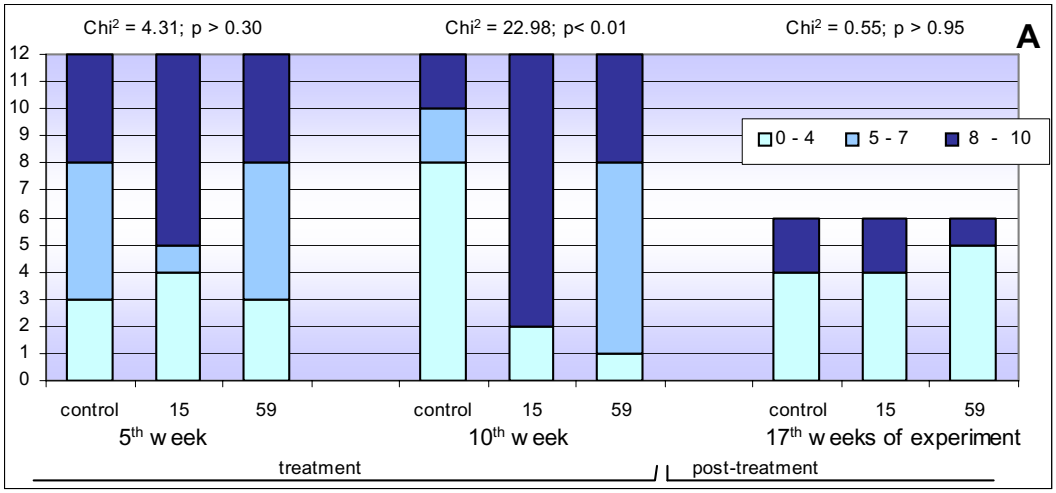
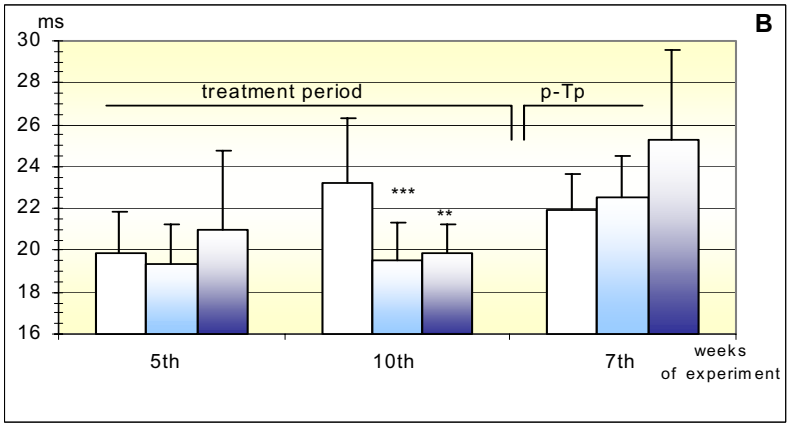


Figure 6 ***A***: Number of responses. The bars show the distribution of rats (n=12/group in the 5th and 10th week of the treatment period [t p], and n=6/group in the 7th week of the post-treatment period [p-t p]) according to the number of noise-positive responses: (0-4, ; 5-7, ; 8-10,) given on 10 consecutive stimuli. ***B***: ordinate: onset latency of noise positive ASR (mean+SD). ***C***: ordinate: peak time (mean+SD) of the responses.
*: p<0.05, **: p<0.01, ***: p<0.001 treated vs. control group
#: p<0.05, ##: p<0.01 high dose group vs. low dose group
Bar pattern: control ; low dose (15 mg/kg) ; high dose (59 mg/kg) .





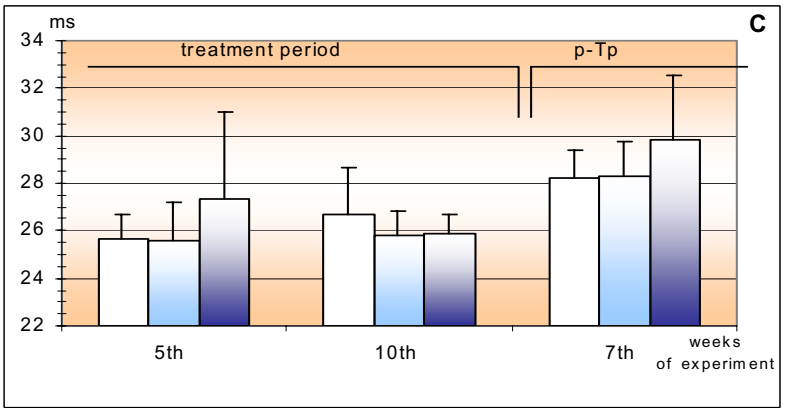


Figure 7 ***A***: Number of responses. ***B***: onset latency and ***C***: peak time of the noise positive ASR of the rats obtained with pre-pulse inhibition (PPI).
A: the bars show the distribution of rats (n=12/group in the 5th and 10th week of the treatment period, and n=6/group in the 7th week of the post-treatment period [p-Tp]) according to the number of noise-positive responses: (0-4, ; 5-7, ; 8-10,) given on 10 consecutive stimuli. *B*: ordinate: onset latency (mean+SD)
C: ordinate: peak time (mean+SD)
**: p<0.01, ***: p<0.001 treated vs. control; else displayed as in Fig. 6.
Bar pattern: control ; low dose (15 mg/kg) ; high dose (59 mg/kg) .

One week after cessation of $MnCl_2$ administration, the average body weight was still significantly reduced in both treated groups ($H_{2;15}$ = 12.02; low dose vs. control: $p<0.01$; high vs. control: $p<0.001$; low vs. high: $p<0.001$). On the 2nd post-treatment week, the two-weeks average body weight of the low dose group approximated that of the control group, but in the high dose group, the body weight difference remained significant in the 6th-7th ($F_{2;3}$ = 75.05; vs. control: $p<0.01$; vs. low: $p<0.01$), 10th-11th ($F_{2;3}$ = 25.99; vs. control: $p<0.01$; vs. low: $p<0.007$), and 12th-13th post-treatment weeks ($F_{2;3}$ = 11.24; vs. control: $p<0.05$; vs. low: $p<0.05$), too.

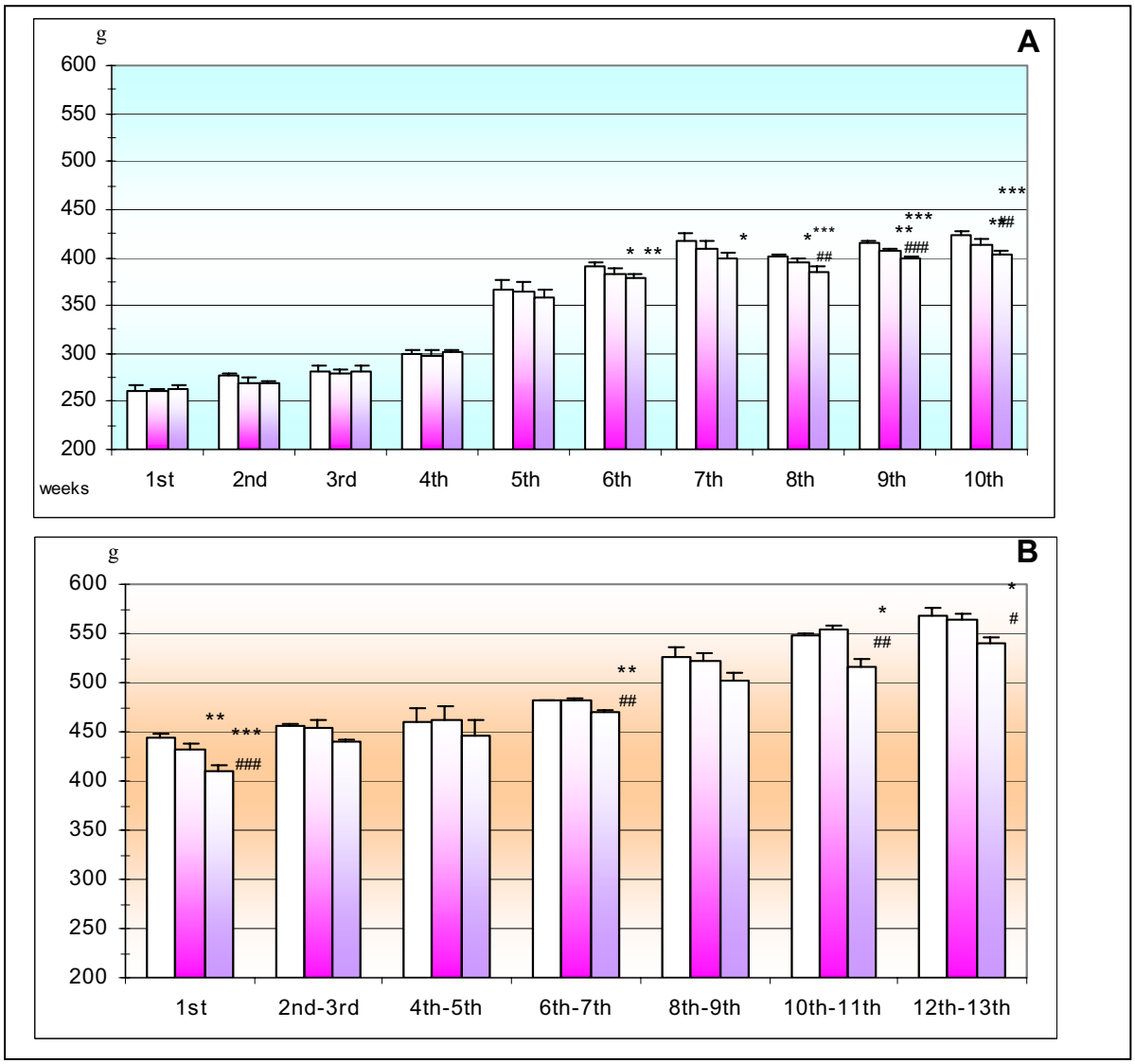


Figure 8 Time course of the rats' body weight. ***A***: During the 1st-10th $MnCl_2$ treatment weeks in control (n=12), low dose (n=10) and high dose (n=10) animals. ***B***: During the 1st –13th post-treatment weeks (n=6 in each group). Ordinate: body weight (g), mean+SD. Bar pattern as in Figure 1 B and C.
*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ treated groups vs. control group
#: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$ high dose group vs. low dose group

4.5.2. Relative organ weight

By the end of the 5th treatment week, relative organ weight was significantly and dose-dependently increased in both $MnCl_2$-treated groups in case of the kidney ($F_{2;6} = 6.41$; control vs. low: $p<0.05$; vs. high: $p<0.05$) and femur (control vs. low $F_{2;6} = 5.58$; vs. high: $p<0.05$; vs. low: $p<0.03$; Table 5). In the low dose group, the relative spleen weight was also elevated ($F_{2;6} = 5.15$; control vs. low: $p<0.05$).

After further 5 weeks of treatment, high dose animals had dose-dependently increased relative spleen ($F_{2;9} = 5.40$; high dose vs. control: $p<0.05$; vs. low dose: $p<0.05$), femur ($H_{2;12} = 5.35$; high dose vs. control: $p<0.05$; vs. low dose: $p<0.05$), and heart ($H_{2;12} = 8.77$; high dose vs. control: $p<0.05$; vs. low dose: $p<0.001$) weights. Significant dose-dependent decrease was seen in the relative adrenal gland weight in the high dose group ($F_{2;9} = 7.21$; high dose vs. control: $p<0.01$), and in the kidney ($F_{2;9} = 5.44$; low dose vs. control: $p<0.01$) and heart ($H_{2;12} = 8.77$; low dose vs. control: $p<0.05$) weight in the low dose group, representing another case of paradoxical dose-dependence.

Table 5 Effects of $MnCl_2$ administration and elimination on the relative organ weights, related to 100 g body weight. (Mean±SD)

Exp.	Weeks of periods	Doses (mg/kg)	Relative organ weights							
			Liver	Lung	Heart	Kidney	Spleen	Thymus	Adr.gland	Femur
Tp	5th (n=3)	Control	3.57 ±0.17	0.55 ±0.18	0.30 ±0.02	0.73 ±0.07	0.16 ±0.02	0.17 ±0.02	0.02 ±0.00	0.19 ±0.01
		15	3.77 ±1.54	0.73 ±0.11	0.35 ±0.05	0.93 ±0.06*	0.22 ±0.03*	0.21 ±0.03	0.01 ±0.00	0.23 ±0.02*
		59	3.23 ±0.42	0.61 ±0.08	0.33 ±0.05	0.91 ±0.09*	0.17 ±0.02	0.20 ±0.03	0.01 ±0.00	0.23 ±0.03*
	10th (n=4)	Control	3.21 ±0.25	0.55 ±0.08	0.30 ±0.01	0.84 ±0.04	0.12 ±0.01	0.14 ±0.03	0.01 ±0.00	0.25 ±0.04
		15	2.97 ±0.27	0.49 ±0.02	0.28 ±0.01*	0.69 ±0.07*	0.13 ±0.01	0.12 ±0.01	0.01 ±0.00	0.24 ±0.01
		59	3.15 ±0.18	0.58 ±0.06	0.32 ±0.02*###	0.74 ±0.08	0.16 ±0.03*#	0.14 ±0.02	0.01 ±0.00**	0.29 ±0.02 *#
p-Tp	12th (n=6)	Control	3.01 ±0.43	0.49 ±0.05	0.27 ±0.03	0.71 ±0.06	0.12 ±0.02	0.10 ±0.01	0.01 ±0.00	0.29 ±0.02
		15	2.77 ±0.34	0.53 ±0.14	0.28 ±0.01	0.70 ±0.05	0.10 ±0.01	0.09 ±0.02	0.01 ±0.00*	0.27 ±0.03
		59	2.86 ±0.21	0.65 ±0.24	0.28 ±0.04	0.71 ±0.05	0.12 ±0.02	0.11 ±0.02	0.01 ±0.00	0.27 ±0.03

Abbreviations: Exp., experimental periods; Tp, treatment period; p-Tp, post-treatment period; n, number of the animals measured/group.
*: $p<0.05$, **: $p<0.01$ treated vs. control group
#: $p<0.05$, ###: $p<0.001$ high dose group vs. low dose group

After 12 weeks without $MnCl_2$ dosing, adrenal gland was the only organ to show significant relative weight difference ($F_{2;15} = 4.42$; control group vs. low dose group: $p<0.01$).

4.6. Immunhistochemistry

In the density of GFAP immunoreactive structures in the hippocampal CA1 region of the animals, no differences were seen between the control animals and those treated with either low or high dose of $MnCl_2$. However, in the hilar part of the DG (Fig. 8), treated animals showed an increase of GFAP-IR density. The effect was significantly dose dependent (control vs. low dose, $p<0.05$; control vs. high dose, $p<0.01$).

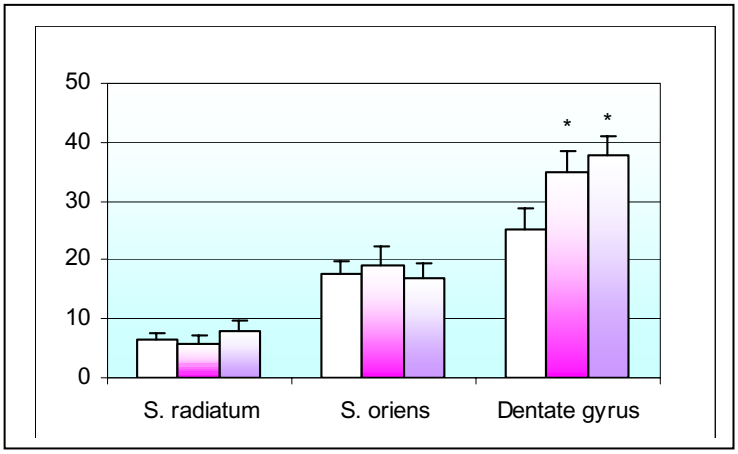


Figure 8 GFAP-IR in the various parts of hippocampus
*: $p<0.05$ treated vs. control group

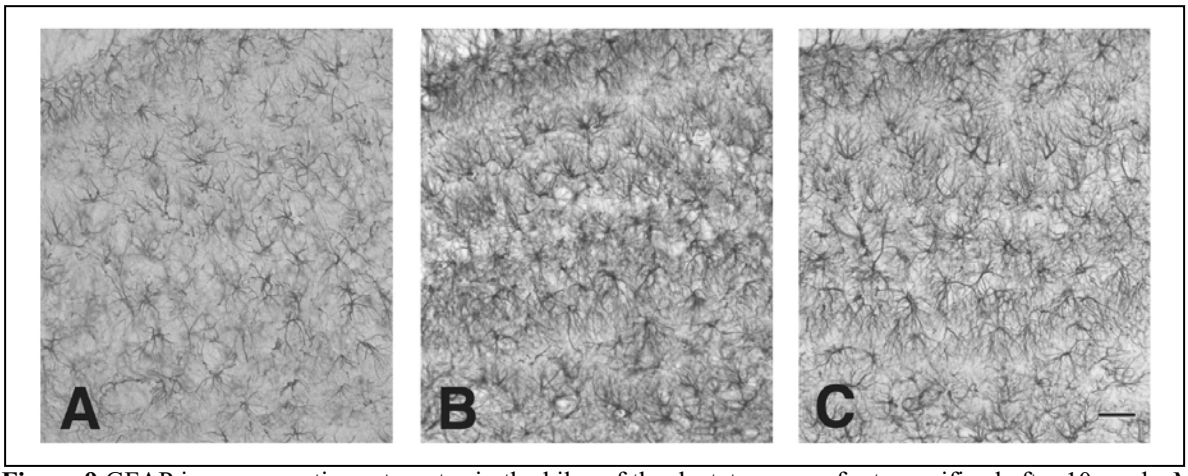


Figure 9 GFAP immunoreactive astrocytes in the hilus of the dentate gyrus of rats sacrificed after 10 weeks Mn treatment. (***A***: control, ***B***: low dose, ***C***: high dose.)
Bar represents 50 μm

5. DISCUSSION

In this complex study, comprising behavioural, morphological and chemical investigations, our aim as to achieve a comprehensive analysis of the alterations evoked by Mn, and, this way, a deep going comparison with data in the literature.

One of the first questions was whether the oral administration of $MnCl_2$ resulted in the required interna exposure. From the rats' gastrointestinal tract, absorption of Mn^{2+} is generally low (<10%) and self-limited (Davis et al., 1992); depending on fullness of the stomach, on the presence of dietary fibres, calcium, phosphorus, phytate, ascorbic acid and copper in the food, as well as on the iron status of the body (Chua and Morgan, 1996), and is also influenced by stressors present in the organism (COMA, 1991).

In humans, the biological half-life of Mn in the body tissues was dependent on the pre-existing body burden, with a shorter half-life seen in workers with higher tissue levels. The clearance half-life of $^{54}MnCl_2$ was about 5 weeks for the body and about 8 weeks for the brain tissue but only a few minutes in the blood (Cotzias et al., 1968).

In our study, blood Mn level was determined in randomly chosen rats in the pre-treatment week, and in the treatment and post-treatment period. In the control rats' blood, brain and other samples, Mn levels (originating from the background Mn present in standard food and drinking water) were similar to that reported by Rehnberg et al. (1982). In the blood, the metal levels were the highest in the 5^{th} treatment week samples, with no significant difference between low dose and control rats. In the high dose group, however, blood, as well as kidney and femur, Mn levels were significantly higher than in the control and low dose group (Table 2 and 3). After another 5 weeks of $MnCl_2$ treatment, the blood Mn level was lower in both treated groups, in the high dose group, 10 times lower than in the 5^{th} treatment week, but the organ Mn levels kept increasing and in the high dose group became significant vs. control. Elevated brain Mn levels were in accordance with the report of Dorman et al. (2000) where 21-day oral exposure to 50 mg/kg $MnCl_2$ increased the striatal, cerebellar and brain Mn concentrations in adult male rats. In other experiments, liver and pancreas were the sites of deposition (COMA, 1991).

In the 6^{th} post-treatment week, blood Mn level in the high dose group was still elevated vs. low dose and control, but was lower than at the end of $MnCl_2$ administration. In

the12th post-treatment week, Mn levels in blood, cortex and HC were no more different in the control and treated rats. In the peripheral tissues of the high dose rats, however, the Mn levels remained higher.

The blood and organ Mn concentrations, and also the organ weights (Table 5), moved differently during the experiment. In the low dose group, Mn levels were not significantly elevated vs. control but the relative weight of the kidney, spleen and femur in the 5th treatment week was higher, and that of the kidney and heart in the 10th treatment week was lower, than in the controls. In the high dose group, Mn levels were higher throughout the whole $MnCl_2$ administration, and the relative weight of kidney and femur in the 5th treatment week, and that of heart, spleen and femur in the 10th treatment week, was also higher than in the controls and low dose.

Mn^{2+}, as a Ca^{2+} antagonist (ILSI, 1994) is known to affect hormone release (Vincent et al., 1985). Altered release of adrenocorticotrophic (ACTH) and/or glucocorticoid hormones causes, on one hand, the shift of Mn from the liver to other parts of the body (Leach and Lilburne, 1978). On the other hand, hypophyseal growth hormone and ACTH act together on the growth of cartilages and bones and of parenchymal organs (Chen et al., 1996). This may be the base of the Mn-induced alteration of relative organ weights (indicating disproportional growth) observed in our study. Damages to the corresponding hypothalamic areas (such as the "hunger centre" in the lateral group of nuclei) by Mn could result in decreased body weight gain (Hume and Egdahl, 1959) as observed in our treated rats during the period of exposure (Fig. 8A and B). In neonatal rats receiving 50 mg/kg $MnCl_2$ per os, Dorman et al. (2000) demonstrated reduced weight gain and increased striatal DA and 3,4-dihydroxyphenylacetic acid levels.

Mn-induced alterations in the dopaminergic system have a secondary endocrine effect due to DA acting on the secretion of pituitary hormones, namely gonadotrophic and growth hormones. In addition to this central effect, DA also acts on the peripheral adrenergic system and has emotional effects. In the 10th week of our treatment, the relative weight of adrenal glands decreased, first of all in the high dose group (vs. control), whereas in the 12th post-treatment week, it increased, and mainly in the low dose animals.

In the high dose group, reduced body weight gain was observed up to the end of the experiment (Fig. 8B) which could also be due to the oxidative damage, i.e. by generation of

reactive oxygen species (ROS; Merry, 2002) caused by Mn effect on the mitochondrial complex II and III (Malecki, 2001; Zhang et al., 2003). On a microinjection of 2 μmol $MnCl_2$ in the striatum, ATP levels were reduced to 51% (vs. control) and lactate level were increased by 97%, indicating the impairment of oxidative energy metabolism (Brouillet et al., 1993).

ROS are also stress factors resulting in increased ACTH release, which in turn is associated with slower motor development, less exploration in a novel environment, and more disturbed behaviour via mainly the hypothalamic-pituitary-adrenal axis (Schneider et al., 1999). ACTH and certain growth factors themselves are known to have behavioural effects (Eliasch, 2001), mediated by the cAMP/cAMP response element binding protein signalling pathways, which have been strongly implicated in the synaptic plasticity associated with long term memory (Bartsch et al., 1998).

In our study, Mn level was significantly elevated after 10 weeks high dose treatment in the cortex and HC (vs. control and low dose group). Elevated hippocampal Mn levels were described after subchronic oral exposure by Dorman et al. (2000), and cortical Mn level increase after chronic treatment, by Takeda et al. (1998).

In the rat brain, there exists a distributed neural axis responsible for behaviour. It comprises the hippocampal formation with functional links between the limbic system, striatum, thalamus (Mair et al., 2002), entorhinal cortex, and basal amygdaloid complex (Groenewegen et al., 1999); the extended hippocampal system, as defined by Aggleton and Sahgal (1993) including the anteroventral thalamic nuclei exhibiting intense AChE staining (Van Groen et al., 1993); as well as other cholinergic systems in the brain (Lydon and Nakajima, 1992). All these play an essential role in spatial learning and memory of rats (Mitchell et al., 2002).

Among the structures mentioned above, the “limbic cortical” subregions (HC, medial prefrontal cortex, and amygdala), as well as the striatum, pallidum or pedunculopontine tegmentum (PPTg): together, the limbic CSPP circuitry (Swerdlow et al., 2001) is important neural substrates for PPI. The limbic CSPP circuitry converges with the primary startle circuit (Lee et al., 1996) at the level of the nucleus reticularis pontis caudalis (PnC). PnC is regarded as a central interface between the acoustic startle and PPI pathways, receiving glutamatergic input from the cochlear nuclei (cochlear root neurons), and cholinergic inhibitory regulation from the PPTg (Leumann et al., 2001).

Reduction of the input of the ascending auditory glutamate pathway in the PnC by Glu antagonists (Krase et al., 1993), or attenuation of the psychomotor stimulant rewarding effects of mesolimbic and mesocortical dopaminergic projections by the selective DA reuptake inhibitor nomifensine given into the nucleus accumbens (Wise, 1998), resulted in reduced ASR. A similar effect of Mn exposure on ASR was described by Dorman et al. (2000). In our experiment, dose dependently diminished magnitude of the ASR was seen in the treated rats in the 10^{th} treatment week, and also in the 7^{th} post-treatment week (Fig. 6A).

The reduced OF activity of the treated rats was probably due to the effect of Mn on the dopaminergic neurons in the ventral tegmental area (Gifkins et al., 2002). The mesolimbic and nigrostrial dopaminergic system (Calabresi et al., 1997), both known to show dysfunction on Mn application, are also involved in the rats' extrapyramidal motor control and (direct or indirect) sensorimotor integration. Depending on the dose and duration of Mn treatment, spontaneous motor activity increased or decreased in rats (Bonilla, 1984), in accordance with the effect of Mn to elicit a biphasic transmitter release response (Eriksson et al., 1987), resulting in e.g. DAergic hyperactivity followed by hypoactivity. Here, the first effect probably results from internal Ca^{2+} mobilization, and the latter, from block of voltage-gated Ca^{2+}-channels. The same was described also in presynaptic Ca^{2+}-channels (Drapeau and Nachshen, 1984). In our experiments, decrease of the spontaneous horizontal and vertical activity developed after 5 weeks, and of the local activity after 10 weeks, of Mn exposure.

By the 7^{th} post-treatment week, there was no more difference between the OF activity of the control and treated rats. In the 10^{th} week of elimination, however, 1.5 mg/kg d-A induced a significant increase of horizontal activity but no alteration in the rearing in the low dose group, while in the high dose group the increase of rearing was higher than that of the horizontal activity. Local activity was strongly decreased in both groups vs. control. Taken together, OF effects indicated the involvement of both the dopaminergic and the serotoninergic transmission in the effects of Mn (as described in the rat striatum, HC and cortex by Subhash and Padmashree, 1991).

The cholinergic input from the PPTg and laterodorsal tegmental nucleus (described with choline acetyltransferase immunhistochemistry; Koch et al., 1993) inhibits the majority of acoustically responsive PnC neurons. Neurotoxic lesion of the cholinergic neurons of the PPTg significantly reduced PPI, without affecting the startle magnitude in the absence of a

pre-pulse. Scopolamine microinfusion to the PPTg dose-dependently enhanced startle and reduced PPI, suggesting that muscarinic receptors in the CnP are involved in inhibition of ASR and the mechanism of PPI (Fendt and Koch, 1999). It was also found in some neuropsychiatric disorders that manipulation or drug-induced changes in these structures resulted in reduced PPI (Braff et al., 1992). Consequently, the inhibition of choline acetyltransferase by $MnCl_2$ in the caudate (Martinez and Bonilla, 1981) and in the striatum (Lai et al., 1981) is a possible explanation of our observation that PPI was significantly reduced (vs. control) at the end of the 10 weeks $MnCl_2$ treatment, but no more after 7 weeks of post-treatment period (Fig. 7).

In the short and long term spatial memory, several of the monoamine neurotransmitters may specifically be involved. Lesions of the NA bundle impaired acquisition (Mason and Fibiger, 1978), damage to the origin of the 5-HT input to the HC disrupted spatial reversal learning and performance in radial maze (Hirata et al., 2001), and the selective destruction of the granule cell population of the DG and of the GABAergic hippocampal interneurons impaired both acquisition and performance in a radial-arm maze (Walsh et al., 1986). DA antagonists disrupt both working and reference memory component of the memory task (Shinotoh et al., 1997).

The hippocampal structure is under central cholinergic inhibitory modulation. Increased mean hippocampal and cortical cholinergic activity plays a role in initial acquisition and retention of the spatial learning task in the 8-arm radial maze (Toumane et al., 1988), regarding working, reference and long term memory (Levy et al., 1991; Lydon and Nakajima, 1992; Paban et al., 2005). Accordingly, a relatively selective cholinergic basal forebrain lesion, and the concomitant massive reduction of choline acetyltransferase activity, caused substantial behavioural deficits (Waite et al., 1995).

The circuits within the HC play a central role in rapidly induced and persistent (NMDA-dependent) synaptic plasticity (Martin and Morris, 2002). The NMDA receptor is required to induce long term potentiation, because Ca^{2+} influx through the NMDA receptor ion channel is the first signal that ultimately changes synaptic strength in the HC (Kentros et al., 1998), and in the DG (Ikegaya et al., 1995). Glutamate, released in the synapses, is taken up by astrocytes and is transformed to glutamine by a Mn-dependent enzyme (Erikson and Aschner, 2005). Being accumulated in the astrocytes, Mn^{2+} inhibits the transformation,

(Normandin and Hazell, 2002), leading to block of NMDA receptors. Excess Glu, or the selective NMDA antagonist 2-methyl-6-[phenylethynyl]pyridine, MPEP (Naie and Manahan-Vaughan, 1991), may interfere with hippocampal NMDA (mGlu 5) receptor function and prevent long term potentiation induction in the CA1 of behaving rats, thus preventing the formation of stable place fields in new and unfamiliar environments, which finally leads to impaired HC-dependent learning and long term working memory in the radial maze (Kentros et al., 1998). In the PnC, NMDA receptors are involved in the modulation of the ASR response (Fendt et al., 1996). The possible outcome, reduced ASR magnitude due probably to tonic inhibition by the excess Glu, was observed in our experiment, too (Fig. 6).

Numerous studies have indicated the importance of the nucleus accumbens (NAC; core and shell subregions; Wan and Swerdlow, 1996) and the mesolimbic dopaminergic system (regulating behaviour via DA-dependent mechanisms; Dawson et al., 1999), and further neurotransmitter systems, such as the intrahippocampal glutamatergic and serotoninergic, and the septohippocampal cholinergic systems (Swerdlow and Geyer, 1998; Wan and Swerdlow, 1996; Koch, 1996), in the normal expression of PPI in rats.

Several of the known or supposed interactions of the major transmitters may have an effect on behaviour and may be affected by repeated or chronic Mn administration. In the behavioural or cognitive functions of the basal forebrain, interactions of acetylcholine (ACh) with Glu (Givens and Sarter, 1997) and GABA (Muir et al., 1992) have been supposed.

DA is known to interact with Glu in the nucleus accumbens and in the nigrostriatal pathway (Konradi, 1998; David et al., 2005); and with GABA in the striato-pallidal projections (Swerdlow et al., 1991), and in the thalamus (Young et al., 1994). The cortico-striatal and limbic glutamatergic-serotoninergic interaction; as well as the muscarinic cholinergic, glutamatergic, dopaminergic and serotoninergic interplay in the sensory and modulatory inputs to PnC (Swerdlow et al., 2001); act as important modulators of PPI in rats (Prinssen et al., 2002). So, excess Mn is known to be involved in the affective and cognitive functions.

The radial maze, used often to test memory functions, is fundamentally based on the utilization of exteroceptive distinction of discriminative stimuli associated to a particular spatial localization (Olton and Samuelson, 1976). This cognitive task has been employed to

investigate the neuronal systems and neurotransmitters involved in learning and memory, and the influence of drugs on these, in rats (Olton, 1987).

In our experiments, the performance in acquisition, and in reference and short term (4h) and long term working memory, a stable and reliable phenomenon in rats, was significantly decreased in the treated animals (Table 3).

In the initial acquisition and retention in a spatial learning task, the cholinergic septohippocampal and magnocellular forebrain-cortical projections, and the DG, which is important in the acquisition of new information and possibly an integral neural substrate for spatial reference and spatial working memory (Eyre et al., 2003), play a major role.

Neurotoxic damage to the granule cells in the DG of the brain of both humans and rodents, detectable histopathologically by immunoreactive staining, can induce significantly more errors during the spatial learning process in the radial maze. In our experiments, gliotic degeneration in the DG and impaired working memory performance were seen together in the 10^{th} treatment week. Selective loss of dentate granule cell populations, leading to persistent impaired acquisition and radial maze performance, and alterations of the ASR, could also be obtained by colchicine injected into DG (Walsh et al., 1986).

To evaluate working memory, retaining information useful for only one session, a delay is introduced in the middle of the task, between the 4^{th} and 5^{th} arm choices, because this increases the complexity of the task. Accordingly, we introduced 2- and 4-hours delays right after acquisition or following a 2-weeks rest period, to measure both short- and long term working memory. In the 5^{th} week, the 4-hours working memory performance was significantly reduced in both treated groups vs. control, and the difference of high vs. low dose group was also significant. At this time, Mn level in the cortex in the high dose rats, and in the HC in both treated groups, was elevated compared to the controls (significance failed because of the low number of animals dissected). In the 10^{th} week, long term working memory deteriorated further in both treated groups vs. control, although significantly elevated Mn levels (vs. control and low dose) were found at this time only in the cortex and HC samples of the high dose rats.

6. CONCLUSION

The main points of conclusion drawn from the above results are as follows:

Oral $MnCl_2$ treatment, in 15 and 59 mg/kg b.w. for 10 weeks, brought about, primarily in the high dose group, an increase of Mn level first in the blood, kidney and femur, later in the liver, cortex and HC as well. Reduced body weight gain and altered relative organ weights were also observed.

During the treatment period, the decrease of 8-arm maze learning performance, the increased gliosis in the hilar part of the hippocampal DG, and alterations of the ASR/PPI magnitude suggested the involvement of the nigrostriatal dopaminergic system and of the ascending auditory glutamatergic and descending cholinergic PPI and/or cortico-hippocampal pathways.

In the post-treatment period, elimination of the metal was first seen from the brain samples, and only later from the peripheral tissues. In the high dose group, the body weight deficit remained throughout the whole experiment. The $MnCl_2$-treated animals' PPI magnitude returned to the control, the alterations of the memory performance and the ASR reaction, however, remained. This indicated a long-lasting effect of $MnCl_2$ on behavioural functions, although the dose-effect relationship in the post-treatment period was, in some cases, anomalous.

By applying a complex set of methods, it was possible to obtain new data for a better-based relationship between the known effects of Mn at neuronal level, and the behavioural and histological outcomes of $MnCl_2$ exposure observed in our study and described in the literature. Taking into account that some of the effects proved to outlast the period of exposure, and the importance of the probably involved systems in the higher-order nervous functions of humans, the neurotoxic risk of environmental and/or occupational $MnCl_2$ exposure seems to deserve ongoing attention.

The alterations seen in our experiment in different elements of the rats' behaviour, when taken together, indicate that the changes were due to the above effects and not to a more general one like ageing.

The results of the presented study can be summarized, corresponding to the points set forth in Introducton and Aims, as follows:

- Oral administration of Mn for 10 weeks resulted in significant decrease of the maze learning performance, OF activity, and the number of ASR responses.
- The majority of the behavioural effects of Mn was detectable already after 5 weeks treatment, and these became more expressed by the 10^{th} week.
- The Mn content in blood, peripheral tissue samples, and in the cortex and HC, increased significantly by the end of the treatment period.
- Some of the Mn-induced behavioural alterations, first of all in the OF, were reversible on cessation of the administration, while others, like decreased working memory and the ASR effects, seemed to be permanent.
- Administration of d-A revealed a lasting effect of Mn on the mechanisms involved in the OF behaviour.

Heavy metals, among them the inorganic form of manganese, the subject of the present study, its organic forms (also investigated but not involved in the Thesis) as well as the inorganic and organic forms of heavy metals are xenobiotics with significant presence in the macro- and microenvironment and corresponding risk populations. This points to the necessity of a sensitive and informative, but non-invasive testing method, especially because a neurotoxic effect can have various indirect consequences. In the studies with manganese presented here, several behavioural functions showed marked or significant alterations. The results of animal experiments cannot be directly transferred to man, but if an experimental approach is complex (including chemical analysis, neurophysiological recording and behavioural tests), standardisable and reveals sensitive markers, it can provide the base of developing methods suitable for population level investigations. Our study, and its planned extensions, can be a contribution to developing such methods.

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9. APPENDIX

In extenso publications

I. *Vezér, T.,* Schulz, H., Nagymajtényi, L.: Memory effect of neurotoxic lead compunds in subacute animal experiments. *Centr. Eur. J. Occup. Environ. Med.,* 6: 209-216 (2000)

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